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Publication date:
1998

Document Version
Publisher's PDF, also known as Version of record

RISØ DK9801566

Risø-R-1042(EN)

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Hydrolysis of Solubilized Hemicellulose Derived from Wet-Oxidized Wheat Straw by a Mixture of Commercial Fungal Enzyme Preparations

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Abstract

The enzymatic hydrolysis of the solubilized hemicellulose fraction from wet-oxidized wheat straw was investigated for quantification purposes. An optimal hydrolysis depends on factors such as composition of the applied enzyme mixture and the hydrolysis conditions (enzyme loading, hydrolysis time, pH-value, and temperature). The concentrated enzyme mixture used in this study was prepared at VTT Biotechnology and Food Research, Finland, by mixing four commercial enzyme preparations. No distinctive pH-value and temperature optima were identified after a prolonged incubation of 24 hours. By reducing the hydrolysis time to 2 hours a temperature optimum was found at 50°C, where a pH-value higher than 5.2 resulted in reduced activity. An enzyme-substrate-volume-ratio of 0.042, a pH-value of 5.0, and a temperature of 50°C were chosen as the best hydrolysis conditions due to an improved monosaccharide yield. The hydrolysis time was chosen to be 24 hours to ensure equilibrium and total quantification.

Even under the best hydrolysis conditions, the overall sugar yield from the enzymatic hydrolysis was only 85% of that of the optimal acid hydrolysis. The glucose yield were approximately the same for the two types of hydrolyses, probably due to the high cellulase activity in the VTT-enzyme mixture. For xylose and arabinose the enzymatic hydrolysis yielded only 80% of that of the acid hydrolysis. As the pentoses existed mainly as complex polymers their degradation required many different enzymes, some of which might be missing from the VTT-enzyme mixture. Furthermore, the removal of side-chains from the xylan backbone during the wet-oxidation pretreatment process might enable the hemicellulosic polymers to interact and precipitate, hence, reducing the enzymatic digestibility of the hemicellulose.

ISBN 87-550-2378-9
ISSN 0106-2840

Information Service Department, Risø, 1998

Contents

Preface

The purpose of this study is to investigate the possibilities of using enzymatic hydrolysis to quantify the solubilized hemicellulose fraction from wet-oxidized wheat straw for evaluation of the efficiency of the pretreatment process as it indicate whether the solubilized hemicellulose is available for fermentation. Previously, acid hydrolysis has been applied for this purpose. However, quantification of the hemicellulose by enzymatic hydrolysis promise several advantages compared to acid hydrolysis. By using enzymatic hydrolysis, the laborious, tedious, and time-consuming sample preparation and purification of the acid hydrolyzates *prior* to HPLC analysis as well as sugar degradation can be avoided.

In this study, the hydrolysis parameters were evaluated, such as the enzyme-substrate volume-ratio, pH-value, temperature, and hydrolysis time, in order to optimize the hydrolysis. For this purpose, a concentrated enzyme mixture was applied prepared at VTT Biotechnology and Food Research, Finland, by mixing four commercial enzyme preparations. This mixture has previously shown promising hydrolysis yields.

Acknowledgment

This study was supported by the Danish Ministry of Energy and Environment (EFP 1383/96-0007) to whom thanks are due. The assistance from Tina Simonsen at Risø National Laboratory in the initial part of the study is gratefully acknowledged. Thanks are also due to VTT Biotechnology and Food Research, Finland, for provinding the enzyme mixture.

1 Introduction

Lignocellulosic substances like wood and agricultural residues are potential raw materials for the production of high-value products. These renewable materials look promising for replacing environmentally unfriendly fossil hydrocarbon materials and for creating "green" products (Ahring *et al.*, 1998; Buttler *et al.*, 1994; Schmidt & Bjerre, 1997).

1.1 Lignocellulose

Cellulose, hemicellulose, and lignin are the main components of plant cell walls, where they tied together form a repelling wall. The wheat straw consists of about 38-45% w/w cellulose, which is the major structural element (Bjerre *et al.*, 1996b; Timell, 1967). Cellulose is a homogeneous polymer and consists of β-1,4-linked glucose units. The polymer may contain up to 10,000 monomers. The cellulose forms long strings that when bound together by hydrogen bonds are called microfibrils. The resistance to tension (stretching) is considered as the primary function of cellulose (Carpita & Gibeaut, 1993).

Lignin is believed to be the most abundant natural aromatic organic polymer (Carpita & Gibeaut, 1993). It is usually located between the cellulose microfibrils, where besides resisting compression forces it also provides protection against consumption by insects and mammals, and also inhibits the rate and degree of microbial degradation (Swain, 1979). Lignin is difficult to study because of its insolubility in most solvents, due primarily to its high molecular weight (probably more than 10,000), but also due to ether bonding to phenolic acids. In wheat straw, these acids bond to the hydroxyl groups of hemicellulose and other polysaccharides by ester linkages in the native state (Bjerre & Schmidt, 1997; Sun *et al.*, 1997).

Hemicellulose is the second most abundant polysaccharide in nature, and accounts for about 33-36% of the dry weight in plant cell walls (Bjerre *et al.*, 1996b; Timell, 1967). Hemicelluloses are referred to mainly as "cellulose"-associated polysaccharides, which can be extracted in alkaline solution (Kong *et al.*, 1992). Unlike cellulose they have a heterogeneous structure consisting of several different monosaccharides depending on the type on material. Hemicellulose molecules are much shorter than cellulose, highly branched, and usually substituted. The most abundant of the hemicelluloses are the xylans (Coughlan & Hazlewood, 1993). Like most polysaccharides, the monosaccharides are linked together by glycosidic bonds. Wheat straw hemicellulose consists mainly of arabino-4-O-methylglucurono xylan with a degree of polymerization of approximately 70 (Coughlan & Hazlewood, 1993).

The xylan molecule consists of a backbone of β-1,4-linked D-xylose molecules (**Figure 1**). The xylanpyranose backbone is usually acetylated in the C-2 and C-3 position. These acetyl-groups are an important barrier against enzymatic hydrolysis (Kong *et al.*, 1992). The xylan is substituted with α-1,2-linked 4-O-methyl-α-D-glucuronic acid. Furthermore, native

xylan has a large content of α-1,3-linked L-arabinosyl side chains. The hemicellulose of wheat straw consists of about 83-85% D-xylose and 15-17% L-arabinose (Bjerre *et al.*, 1996b; Fidalgo *et al.*, 1993). Some of the L-arabinosyl residues are esterified with ferulic and p-coumaric acids, which can form dimers by crosslinking the xylan molecules. The ferulic and p-coumaric residues may also be involved in linking xylan to lignin, since those residues also are present in lignin.

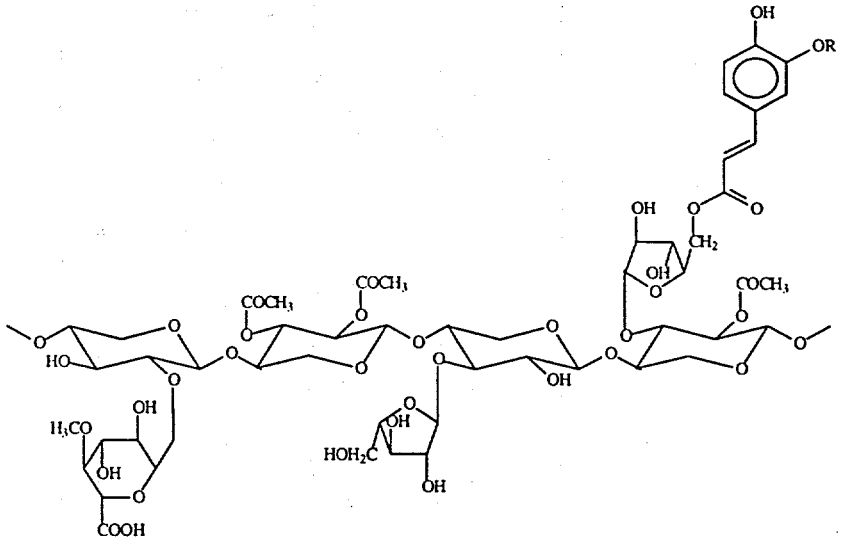

Figure 1. Proposed structure of wheat straw (grasses) xylan (Puls & Schuseil, 1993). (ferulic acid (R = OCH_3), p-coumaric acid (R = H))

1.2 Pretreatment

Due to the rigid and compact structure of plant cell walls, pretreatment is necessary *prior* to enzymatic hydrolysis and fermentation of the polysaccharides. Without pretreatment the hydrolysis and fermentation rates are very low because of limited accessibility of the hemicellulose. This is due to factors such as specific surface area, fiber porosity, fiber pore size, and the presence of other cell wall components (Viikari *et al.*, 1994). In the native state, xylan is part of an insoluble matrix containing cellulose, pectin, and lignin (Carpita & Gibeaut, 1993).

Different pretreatment processes (**Table 1**) have been found efficient for fractionating different types of lignocellulosic materials, making the hemicellulose accessible (Saddler *et al.*, 1993; Schmidt *et al.*, 1996). Physical pretreatments can be classified as mechanical and non-mechanical. The purpose of mechanical pretreatment is to separate the lignocellulosic material into smaller particles, making it more accessible to enzymatic hydrolysis. Ball milling is an example of a mechanical pretreatment method and although effective the pretreatment time and processing cost make ball milling impractical on a large production scale (Fan *et al.*, 1982).

Chemical pretreatment of lignocellulose is widely used in the paper industry to remove hemicellulose and lignin from the cellulose. The chemicals used for pretreatments can be divided into alkalis, acids, and

gases. Acid hydrolysis is an effective method for total or partial hydrolysis of lignocellulosic materials to monomeric saccharides. The disadvantage of using acids is the required removal of acid *prior* to enzymatic or biological utilization. Alkali treatment results in an increased swelling in water, making lignocellulosic materials more accessible to enzymatic hydrolysis; however, the lignin content will determine the efficiency of the pretreatment (Fan *et al.*, 1982). Pretreatment with gases has the advantage that it is throughout the material yielding a uniform product. However, the difficulties in reusing chemicals and the long treatment time (2-24 hours) make the use of gases in a pretreatment process quite expensive (Fan *et al.*, 1982).

Table 1. Methods for pretreating lignocellulosic materials to enhance enzymatic hydrolysis (Fan et al., *1982; Saddler* et al., *1993).*

Physical	Chemical	Biological	Combination
Steaming	Hydrochloric acid	White rot fungi	Steam explosion
Radiation	Sulfuric acid		Wet oxidation
Wetting	Phosphoric acid		Milling at high temperatures
Ball milling	Acetic acid		Alkali and ball milling
Hammer milling	Sodium hydroxide		
Milling	Ammonia		Sulfur dioxide and steaming
Vibrator rod	Sodium carbonate		
	Sulfur dioxide		Nitrogen dioxide and irradiation
	Oxygen		

Combined pretreatment methods such as steaming and other hydrothermic processes have been found efficient for increasing the digestibility of lignocellulose by enzymatic hydrolysis (Fan *et al.*, 1982; Saddler *et al.*, 1993). The wet-oxidation process (water, oxygen and elevated temperatures) combined with alkaline hydrolysis efficiently dissolved the hemicellulose from wheat straw, whereas cellulose and some lignin remain insoluble (Bjerre *et al.*, 1996b; Schmidt & Thomsen, 1998). During the steam explosion process 2-furfural and 5-hydroxymethyl-2-furfural are formed. Both of these are undesirable because of their inhibitory effect on microorganisms (Buchert, 1990).

To evaluate the efficiency of a pretreatment process enzymatic hydrolysis can be performed, which will indicate how accessible the hemicellulose is for enzymes, and hence, how easily it can be fermented to high-value products (Bjerre & Schmidt, 1997). The enzymatic hydrolysis can either be a step in the overall process from lignocellulose to high-value products or an analytical tool to quantify solubilized hemicellulose.

2 Hemicellulose-degrading Enzymes

The complete enzymatic break down of a complex hemicellulose structure requires the action of several hydrolytic enzymes. The enzymes hydrolyzing hemicelluloses are called hemicellulases and are composed of endo-enzymes that cleave internal glycosidic bonds, exo-enzymes that remove sugar residues from the non-reducing end of oligosaccharides, and esterases that attack non-glycosidic ester linkages (**Figure 2**).

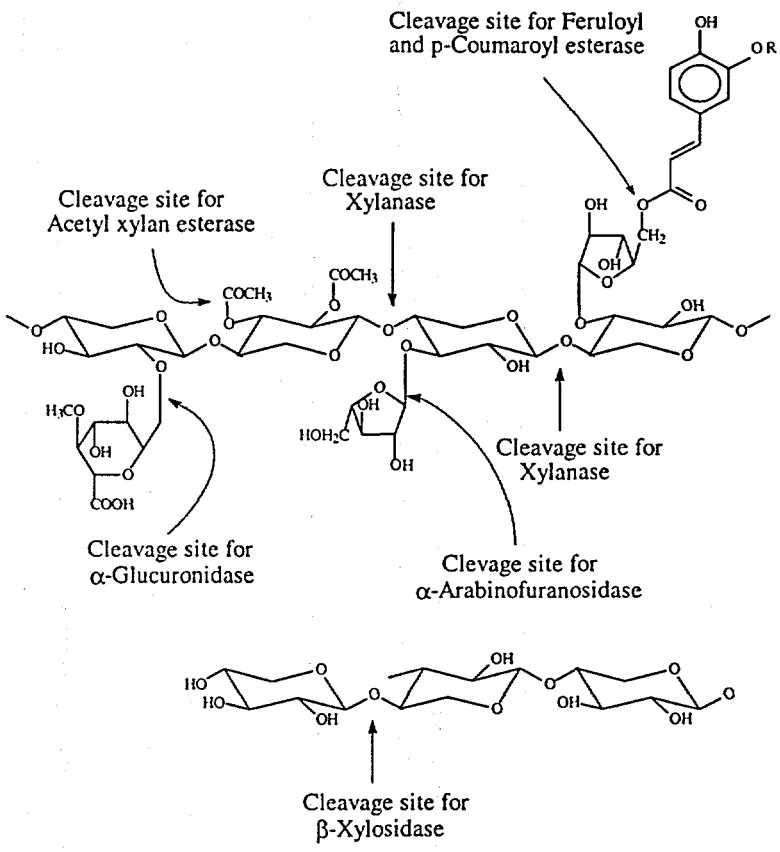

Figure 2. The cleavage sites for the hemicellulosic enzyme system on the proposed structure of wheat straw xylan (Biely, 1993; Puls & Schuseil, 1993). (ferulic acid (R = OCH_3), p-coumaric acid (R = H))

The biosynthesis of xylan-degrading enzymes by xylanolytic microorganisms with xylan as carbon source is well-documented (Coughlan *et al.*, 1993) The heavily substituted soluble xylans are too large to be transported into the cells of the microorganisms; hence, xylanase biosynthesis is induced by xylan via the soluble low-molecular catabolites generated from xylan by the action of extracellular or cell-membrane-associated xylan-degrading enzymes (Coughlan & Hazlewood, 1993). Xylobiose induces the synthesis of xylan-degrading enzymes in a number of microorganisms, where the most potent inducer is β-1,3-xylobiose. However, the conversion of xylan to xylose might also lead to inhibition via catabolic repression (Hrmová *et al.*, 1991).

The enzyme system exists in a vast amount of microorganisms such as bacteria (Berenger *et al.*, 1985; Esterban *et al.*, 1983; Nakajima *et al.*,

1984), yeast's (Biely *et al.*, 1980) and fungi, where the fungi *Aspergillus* and *Trichoderma* are the ones most commonly used for industrial production of hemicellulases due to the extracellular nature of the enzymes with a broad substrate specificity (Thomson, 1993). *Trichoderma reesei* is one of the most powerful producers of hemicellulases, and a vast number of xylanases, β-xylosidases, α-glucuronidase, and acetyl esterases have been isolated and purified (Tenkanen, 1995).

2.1 Endo-β-1,4-Xylanase (EC 3.2.1.8)

The key enzyme in the xylan degradation is the endo-β-1,4-xylanase, which attacks the xylan backbone randomly by hydrolyzing the β-1,4-D-xylosidic linkage (**Figure 2**). For *Aspergillus*, the rate of hydrolysis was found to be directly proportional to the chain length of the xylan oligosaccharides, where the activity toward xylobiose and xylotriose was found to be negligible (Duarte & Costa-Ferreira, 1994). The main hydrolysis products are substituted and non-substituted oligomers containing 2-4 xylose monomers.

Endo-xylanases of fungal origin have molecular weights in the range of 7-60 kDa and generally show the highest activity at pH 3.5-6.0 and 40-60°C (Kormelink, 1992). *Aspergillus niger* and *T. reesei* produce, like other xylanolytic microorganisms, more than one xylanase. Two main xylanases have been isolated from *T. reesei* with a pI of 5.5 and 9.0 (Tenkanen *et al.*, 1992a; 1992b). Their pH-optimum ranges from 4 to 5.5.

Five different xylanases have been isolated from *A. niger* which differs in physical and chemical properties such as optimal reaction conditions and preferred cleavage sites (Wong *et al.*, 1988). Their pH-optimum lies between 4-6 and the temperature-optimum between 50-55°C similar to those of *T. reesei*. Xylanases are generally considered to be quite stable, and stability tests show that only a minor activity is lost when incubated at 50°C for 200 minutes (Lahaye & Vigouroux, 1992).

2.2 β-D-Xylosidase (EC 3.2.1.37)

The exo-β-D-xylosidase hydrolyzes non-substituted xylo-oligo-saccharides to xylose (**Figure 2**) by cleaving the same β-1,4-D-xylosidic linkage as the endo-xylanases. Thus being an exo-enzyme, only non-reducing ends of the oligosaccharides are attacked. β-D-Xylosidase shows the highest activity toward xylobiose and the activity decreases with increasing chain length. The main hydrolysis product is xylose, which inhibits the action of the β-xylosidase in a competitive manner by confining itself to the active site of the enzyme (Thomson, 1983).

The β-D-xylosidase from *T. reesei* is unable to hydrolyze substituted xylo-oligosaccharides, such as arabinose-substituted or 4-O-methyl-glucuronosyl-substituted xylo-oligosaccharides (Coughlan & Hazlewood, 1993). The enzyme cleaves off all unsubstituted xylose units from the non-reducing end of 1,2-linked uronic acid substituted xylo-oligo-

saccharides (Tenkanen *et al.*, 1996). Furthermore, arabinose-substitution was found to protect the β-1,4-xylosidic linkage before the substituted xylose unit (Tenkanen *et al.*, 1996). In addition to p-nitrophenyl-β-xylopyranoside and non-substituted xylo-oligosaccharides, the β-xylosidase of *T. reesei* also shows activity toward p-nitrophenyl-α-arabinofuranoside, but can not hydrolyze arabinans or release arabinose from arabinoxylans (Poutanen & Puls, 1989). The β-D-xylosidases has pH-optimum at around 4.0 with temperature optimum around 60°C (Poutanen & Puls, 1988).These values are only slightly different to the β-D-xylosidases from *A. niger*, which has a half-life of about 75 h at 65°C and pH of 4.2 (Coughlan *et al.*, 1993).

2.3 Arabinose-Releasing Enzymes

The arabinosidase enzymes belong to the side-group-cleaving enzymes, and hydrolyze α–L-arabinofuranosyl groups of arabino-hemicelluloses yielding arabinose (**Figure 2**). Three types of arabinose-releasing enzymes have been identified from *A. niger* and *A. aculeatus*. Two α-L-arabinofuranosidases were found to hydrolyze the α-1,2- and α-1,3-glycosidic bonds which differ in substrate specificity (Rombouts *et al.*, 1988). An endo-1,5- α-L-arabinanase able to hydrolyze α-1,5-glycosidic bonds was also found (Rombouts *et al.*, 1988). Additionally, a 1,4-β-D-arabinoxylan arabinofuranohydrolase has been isolated from *A. awamori* with a high substrate specificity (Kormelink *et al.*, 1991).

2.3.1 α-L-Arabinofuranosidase (EC 3.2.1.55)

Arabinofuranosidases are the main side-group-cleaving enzymes regarding wheat straw hemicellulose. Two different enzymes have been identified. Arabinofuranosidase A and B were both able to hydrolyze p-nitrophenol-arabinofuranoside and α-1,5-L-arabinofuranose oligomers. Arabino-furanosidase B was active toward arabinoxylan whereas arabino-furanosidase A was inactive toward arabinoxylan as it was incapable of splitting 1,3-α-L- or 1,2-α-L-linked arabinose substituents (Rombouts *et al.*, 1988). Additionally, arabinofuranosidase B showed activity toward terminal arabino-substituted xylan (Rombouts *et al.*, 1988). In comparison to xylanases, the molecular weights of arabino-furanosidases are high, the optimal pH-value varies between 3.7 and 5.0 for enzymes produced by *T. reesei* and *A. niger* with optimal temperatures around 50-60°C (Poutanen, 1988; Rombouts *et al.*, 1988).

2.3.2 Endo-1,5-α-L-arabinanase (EC 3.2.1.99)

Endo-1,5-α-L-arabinanase hydrolyzes 1,5-α-L-arabinofuranosidic linkages in arabinans. The arabinanase isolated from *A. niger* was highly active toward linear arabinan and to a lesser degree branched arabinan and arabinogalactan as well as activity against arabinoxylan (wheat, oat spelt). The action of arabinanase is inhibited by the presence of arabinofuranosyl branches (Beldman *et al.*, 1993). The activity of the

arabinanase from *A. niger* was optimal at pH 5 and 50°C (Rombouts *et al.*, 1988) compared to the one from *A. aculeatus*, which had an optimum pH-value of 5.5 and was stable only in a narrow pH-range (5.5-6.3) (Beldman *et al.*, 1993).

2.3.3 Arabinoxylan arabinofuranohydrolase

Recently, an additional arabinofuranosidase has been identified which differs in substrate specificity. 1,4-β-D-Arabinoxylan arabinofuranohydrolase has been isolated from *A. awamori* (Kormelink *et al.*, 1991). The enzyme displays a high substrate specificity against arabinoxylans, but shows no activity toward p-nitrophenol-arabinoside, arabinans and arabinogalactans. The arabinofuranohydrolase had an optimal pH-value of 5.0 and a temperature optimum of 50°C (Kormelink *et al.*, 1991)

2.4 α-Glucuronidase (EC 3.2.1.31)

The α-glucuronidase (**Figure 2**) hydrolyzes the α-1,2-glycosidic bonds between xylose and either D-glucuronic acid or 4-O-methyl-D-glucuronic acid (Thomson, 1993). Apart from acetyl and arabinosyl side-groups, glucuronic acid is the most abundant side-group in native xylan and plays an important role in the enzymatic degradation, where it inhibits the complete enzymatic conversion of xylan into monosaccharides (Puls *et al.*, 1987). Two different α-glucuronidases have been isolated from *A. niger* with an optimal temperature and pH-value of 60°C and 4.8 for both enzymes (Uchida *et al.*, 1992). Incubation of glucuronoxylan with some α-glucuronidases alone does not yield glucuronic acid indicating an inability of these enzymes to act on native xylan; however, they act synergistically with endo-xylanases (Puls *et al.*, 1987).

2.5 Acetyl Esterases

Esterases that deacetylate acetylxylan (**Figure 2**) are produced by many fungi, including *A. niger* and *T. reesei*. The most important esterases are acetylxylan esterase (EC 3.1.1.72) and acetyl esterase (EC 3.1.1.6). A distinction between acetylxylan and acetyl esterase is made by their ability to act on native xylan. Acetylxylan esterase releases acetic acid from xylan in the absence of endo-xylanases, whereas acetyl esterase needs the cooperative action of endo-xylanases (Biely *et al.*, 1986). Some acetyl esterases display a wide substrate specificity cleaving bonds other than acetyl ester linkages (Tenkanen *et al.*, 1991). The esterases act synergistically with xylanase by removing the acetyl groups from acetylated xylan as the presence of acetyl constituents impedes the activity of the endo-xylanases (Biely *et al.*, 1986). Acetylxylan esterase of *A. niger* has a pH-optimum of 7.0 and a temperature optimum at 40-50°C (Kormelink, 1992; Lindén *et al.*, 1994), whereas the values for acetylxylan esterase of *T. reesei* are 5.0-6.0 and 50-65°C, respectively (Sundberg & Poutanen, 1991). These esterases might not play a key role in the hemicellulose degradation of wet-oxidized wheat straw, since the

hemicellulose probably is deacetylated during the process (Bjerre *et al.*, 1996b) as indicated by the observed loss in ester bonds determined by FT-IR analysis of wet-oxidized wheat straw (Schmidt *et al.*, 1998). However, this needs further investigation.

2.6 Phenolic Acid Esterases

Ferulic and p-coumaric acids are esterified to arabinofuranosyl units of the arabinoxylan (**Figure 2**), hence, feruloyl and p-coumaroyl esterases play an important role in the initial degradation by cleaving the cross-linkage between xylan chains and between xylan and lignin parts (Mueller-Harvey *et al.*, 1986).

T. reesei is known to produce a wide variety of xylanolytic enzymes; however, no esterase able to liberate ferulic acid from wheat straw xylan has been found (Tenkanen *et al.*, 1991). Different *Aspergillus* strains such as *A. awamori*, *A. niger*, *A. oryzae* and *A. terreus* are known to produce such an esterase (Poutanen & Puls, 1989). The type of growth medium affected the type of feruloyl esterase produced by *A. niger* (Faulds & Williamson, 1993), but the production seemed to be unrelated to the ferulic acid contents in the medium (Tenkanen, 1995). Three different types of feruloyl esterases have been isolated from *A. niger*.

Two esterases with feruloyl esterase activity have been purified from *A. oryzae* (Tenkanen *et al.*, 1991). The esterases were of low molecular weight and had a pH-optimum at around 5.0 and temperature-optimum of 45°C. Since acetyl esterases has a wide substrate specificity it is difficult to distinguish its activity from that of specific feruloyl and p-coumaroyl esterases. Like acetyl xylan esterase the feruloyl esterase acts synergistically with endo-xylanase (Faulds & Williamson, 1991). The cleavage site for the esterases was hindered in native lignocellulose due to the compact structure of lignocellulose; hence, the presence of Celluclast (Novo Nordisk, Denmark) solubilized the cellulose and to some extent also the xylan making the cleavage site available to the esterases (McCrae *et al.*, 1994).

2.7 Summary

The complete enzymatic degradation of hemicellulose derived from wheat straw requires the action of a wide range of enzymes (**Table 2**). The complex nature of the enzyme system involved in the degradation of lignocellulosic materials can be illustrated by synergistic effects present in the different side-group-cleaving enzymes in the system. Acetyl esterase is needed to remove most of the acetyl groups for the endo-xylanase, effectively degrading the xylan backbone to less-substituted xylo-oligosaccharides. Subsequently, the different side-group-cleaving enzymes, like arabinofuranosidase, glucuronidase, feruloyl, and coumaroyl esterase will be able to attack the xylo-oligomers, thereby removing the side-groups. The unsubstituted oligomers are then hydrolyzed to xylose by β-xylosidase.

Table 2. Hemicellulolytic enzymes from Aspergillus niger *and* Trichoderma reesei *and their typical properties.*

EC-number	Enzyme	Substrate	Linkage hydrolyzed	pH-optimum	Temperature-optimum
3.2.1.8	Endo-1,4-β-xylanase	Xylan backbone	Endo–β-1,4 glycosidic bond	4.5 - 6.0	40 - 60°C
3.2.1.37	β-D-xylosidase	Xylan oligomers	Exo–β-1,4 glycosidic bond	3.0 - 5.0	40 - 60°C
3.2.1.55	α-L-Arabinofuranosidase	Arabinose groups	α-1,2 glycosidic bond α-1,3 glycosidic bond α-1,5 glycosidic bond	3.8 - 4.0	60°C
3.2.1.99	Endo-1,5-α-arabinanase	Arabinose groups	α-1,5 glycosidic bond	5.0	50°C
3.2.1.?	Arabinoxylan-Arabinofuranohydrolase	Arabinose groups	α-1,4 glycosidic bond	5.0	50°C
3.2.1.31	α-Glucuronidase	Glucuronic acid groups	α-1,2 glycosidic bond	4.8	60°C
3.1.1.72	Acetyl xylan esterase	Acetyl groups	Ester bonds	5.5 - 7.5	45 - 55°C
3.1.1.6	Acetyl esterases	Acetyl groups	Ester bonds	5.0 - 7.0	45 - 65°C
3.1.1.?	Feruloyl esterase	Feruloyl groups p-Coumaroyl groups	Ester bonds	4.5 - 6.0	45°C

2.8 Aims

The purpose of this study is to investigate the possibilities of using enzymatic hydrolysis to evaluate the efficiency of the pretreatment process. Enzymatic hydrolysis can indicate whether the solubilized hemicellulose is available for fermentation, or can be employed as a quantification tool for solubilized hemicellulose. The quantification of the hemicellulose by enzymatic hydrolysis will yield several advantages compared to acid hydrolysis, which is presently employed (Bjerre *et al.*, 1996a). By using enzymatic hydrolysis, the laborious, tedious, and time-consuming sample preparation and purification of the acid hydrolyzates *prior* to HPLC analysis can be avoided. In this study, the hydrolysis parameters were evaluated, such as the enzyme-substrate volume-ratio, pH-value, temperature, and hydrolysis time, in order to optimize the enzymatic hydrolysis of wet-oxidized wheat straw. For this purpose, a concentrated enzyme mixture of 4 commercial enzyme preparations (Econase, Ecopulp, Gamanase, Novozym) was applied, prepared by VTT Biotechnology and Food Research, Finland.

3 Materials and Methods

3.1 Enzymes

The concentrated enzyme mixture was prepared at VTT Biotechnology and Food Research, Finland, by combining commercial cellulase (Econase, Alko Ltd., Finland), xylanase (Ecopulp, Alko Ltd., Finland), mannanase (Gamanase, Novo Nordisk A/S, Denmark) and β-glucosidase (Novozym, Novo Nordisk A/S, Denmark) preparations. The Econase and Ecopulp were produced by a strain of *Trichoderma reesei* whereas Gamanase and Novozym were produced by *Aspergillus*. The mixture of the four commercial enzyme preparations was gel filtrated though a P-6 column (Biorad, USA) in order to remove contaminating sugars (Tenkanen *et al.*, 1995b). The different cellulolytic and hemicellulolytic enzyme activities of the final mixture (**Table 3**) were assayed according to previously reported methods (Bailey *et al.*, 1992; IUPAC, 1987; Poutanen, 1988; Rättö & Poutanen, 1988; Siika-aho *et al.*, 1994).

Table 3. The activities in the VTT-enzyme mixture (Data provided by VTT Biotechnology and Food Research, Finland).

	Enzyme	Activity	Units
FPU[a]	FPase (Cellulase)	49	U/mL
HEC[b]	Endo-glucanase	4850	nkat/mL
XYL	Xylanase	5360	nkat/mL
MAN	Mannanase	10250	nkat/mL
β-Glu	β-Glucosidase	2050	nkat/mL
β-Xyl	β-Xylosidase	240	nkat/mL
β-Man	β-Mannosidase	71	nkat/mL
α-Ara	α-Arabinofuranosidase	1000	nkat/mL
α-Glur	α-Glucuronidase	2	nkat/mL
α-Gal	α-Galactosidase	240	nkat/mL

a: Filter Paper Activity Units
b: HEC: Hydroxyethylcellulose

3.2 Pretreatment

The substrate used for optimizing the enzymatic hydrolysis was produced at Risø National Laboratory by wet oxidation in a specially designed loop-autoclave constructed at Risø National Laboratory (Bjerre *et al.*, 1996b) with a working volume of 1 L. Wheat straw from 1993 was mixed with water and Na_2CO_3 before applying the oxygen pressure and subsequently the heat (Bjerre *et al.*, 1996b). The conditions used are given in **Appendix A**. After pretreatment, the suspension was filtered to separate the solid cellulose-rich fraction from the liquid hemicellulose-rich fraction. The filtrate containing the solubilized hemicellulose was employed as substrate in the succeeding hydrolysis study.

3.3 Enzymatic Hydrolysis

3.3.1 Enzyme-substrate-volume-ratio

The hydrolysis reaction mixture was created by mixing wet oxidized substrate and the VTT-enzyme mixture with 0.1 M acetate buffer (pH 5.0). To obtain different enzyme-substrate-volume-ratios, the volume of both VTT-enzyme mixture and substrate was varied, whereas the amount of buffer was kept constant (**Table 4**). To achieve a 1-mL hydrolysis reaction mixture, the total volume of VTT-enzyme mixture and substrate was 250 µL mixed with 750 µL of acetate buffer.

Table 4. Calculation of the different enzyme-substrate-volume-ratios.

Code	VTT-enzyme (µL)	Substrate (µL)	Volume-ratio	Volume-ratio	Xylanase-ratio[a] (nkat/mg)
EH2	2	248	1/124	0.008	1,541
EH5	5	245	1/49	0.020	3,900
EH10	10	240	1/24	0.042	7,962
EH20	20	230	2/23	0.087	16,616
EH30	30	220	3/22	0.136	26,057
EH50	50	200	1/4	0.250	47,772

a: Xylanase activity per g dry weight of substrate
The dry weight of substrate was 28 mg/mL (B89)

The hydrolysis mixture was incubated for 24 hours at 45°C in a heated lab shaker (Adolf Kühner AG). The reaction was stopped by heating at 100°C for 5 minutes followed by centrifugation. The supernatant was diluted with deionized water (3:4) and analyzed for monosaccharide composition by HPLC.

3.3.2 Reaction time

The hydrolysis mixture was incubated at 50°C and pH 5.0 using the best enzyme-substrate-volume-ratio for varied hydrolysis time ranging from 0.25 to 24 hours. The hydrolysis mixture consisted of 750 µL acetate buffer, 240 µL substrate and 10 µL VTT-enzyme mixture.

3.3.3 pH-value

Different 0.1 M acetate buffers were created by mixing varying amounts of sodium acetate and acetic acid to obtain different pH-values in the hydrolysis mixture. The 1-mL hydrolysis mixture consisted of 750 µL acetate buffer, 240 µL substrate and 10 µL VTT-enzyme mixture. The samples were incubated for 2 or 24 hours at 45°C or 50°C.

3.3.4 Temperature

The hydrolysis mixture was incubated for 24 hours at different temperatures ranging from 30 to 50°C in the heated lab shaker. The hydrolysis mixture consisted of 750 µL acetate buffer, 240 µL substrate and 10 µL VTT-enzyme mixture. Temperatures exceeding 50°C were not possible using this equipment. Hence, further experiments were carried out in a water bath at temperatures from 30 to 70°C for 2 hours.

3.4 Acid Hydrolysis

The acid hydrolysis was performed at 121°C with 4% w/v H_2SO_4 for 10 minutes (Bjerre *et al.*, 1996a). After hydrolysis the solutions were filtered in order to remove the water-insoluble residue. In order to avoid interference from salts and non-sugar substances in the HPLC analysis with subsequently damage to the column, acid hydrolyzates need to be deionized *prior* to injection (Kaar *et al.*, 1991; Puls, 1993). A combination of precipitation and ion exchange was applied (Bjerre *et al.*, 1996a).

3.5 Monosaccharide Quantification

The monosaccharides were measured by HPLC using an Aminex HPX-87H column with a Biorad pre-column at 63°C. The eluent used was 0.004 M H_2SO_4 at a 0.6 mL/min. flow rate (Bjerre *et al.*, 1996a). The monosaccharides were detected by their differential refractometer index. The measured monosaccharides were glucose (R_t=8.6), xylose (R_t=9.2), arabinose (R_t=10.0), and acetic acid (R_t=14.4).

4 Results and Discussion

In order to compare acid and enzymatic hydrolysis of wet-oxidized wheat straw, the conditions under which the hydrolyses were performed had to be investigated. The acid hydrolysis has previously been optimized for wet-oxidized wheat straw (Bjerre *et al.*, 1996a), whereas this study focuses on optimizing the enzymatic hydrolysis.

4.1 Effect of Enzyme-Substrate-Volume-Ratio

To obtain an effective enzymatic hydrolysis of a complex substrate like wheat straw hemicellulose, the specific activity of all essential enzymes has to be present at a satisfying level. This is particularly important as many of the enzymes act synergistically. In order to determine the influence of enzyme-substrate-volume-ratio the volume of both VTT-enzyme mixture and substrate were varied, whereas the amount of acetate buffer was kept constant (**Figure 3**).

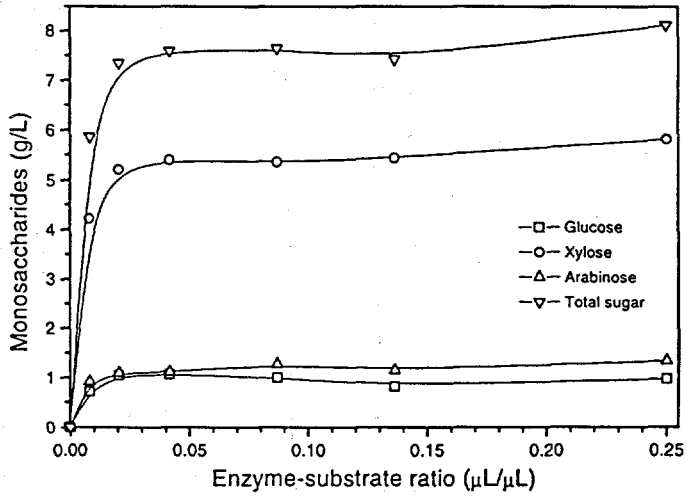

Figure 3. Monosaccharide yield after enzymatic hydrolysis of wet-oxidized wheat straw hemicellulose using the VTT-enzyme mixture as a function of enzyme-substrate-volume-ratio (as given in ***Table 4****) (Conditions: 45°C, 24 hours, pH 5.0, substrate (B89)).*

Even a very low amount of the VTT-enzyme mixture was sufficient for the hydrolysis of wet-oxidized wheat straw hemicellulose (**Figure 3**), although a minimal volume-ratio of 0.02-0.04 was needed. The optimal volume-ratio was achieved by adding 10 µL VTT-enzyme mixture to 240 µL wet-oxidized wheat straw giving a volume-ratio of about 0.04. This volume-ratio give a xylanase activity of nearly 8,000 nkat/g dry weight of substrate (**Table 4**). Using 5 µL VTT-enzyme mixture (a volume-ratio of 0.02, xylanase activity of 4,000 nkat/g dry weight of substrate) gave nearly as good a hydrolysis as the 10 µL, but for analytical purposes 5 µL was difficult to handle, and hence the 10 µL was used. However, for a

larger production scale the low enzyme-substrate-volume-ratio should be used for economic reasons. A slight increase in hydrolysis was observed at 50 µL VTT-enzyme mixture (a volume-ratio of 0.25) but not significant to justify such a larger enzyme dose (48,000 nkat/g).

4.2 Effect of Reaction Time

In order to determine the optimal reaction time, an experiment was conducted using different reaction times as shown in **Figure 4**. Even though 16 hours were sufficient to obtain maximal hydrolysis, a reaction time of 24 hours was chosen in order to ensure equilibrium and total quantification. Already after about 2-5 hours of hydrolysis nearly all the sugars have been released from the polymer (about 90%). Hence, at production scale this considerably shorter reaction time would be possible without encountering a great loss of monosaccharides that improve the economy of the hydrolysis process. Glucose, which is derived from cellulose, was liberated very rapidly indicating that cellulose-degrading enzymes were present in the VTT-enzyme mixture in high amount (**Table 3**). The hydrolysis of the main hemicellulose chain was considerable slower (release of xylose) than those of the side-chain (release of arabinose). This might indicate that missing/limiting activity of some side-chain cleavage enzymes led to steric hindering of the xylosidase and xylanase enzymes giving a slower hydrolysis of the xylan main-chain (Coughlan *et al.* 1993). The slower hydrolysis of wet-oxidized hemicellulose might also be due to a tighter conformation of the hemicellulose as some precipitation of hemicellulose has been observed during storage.

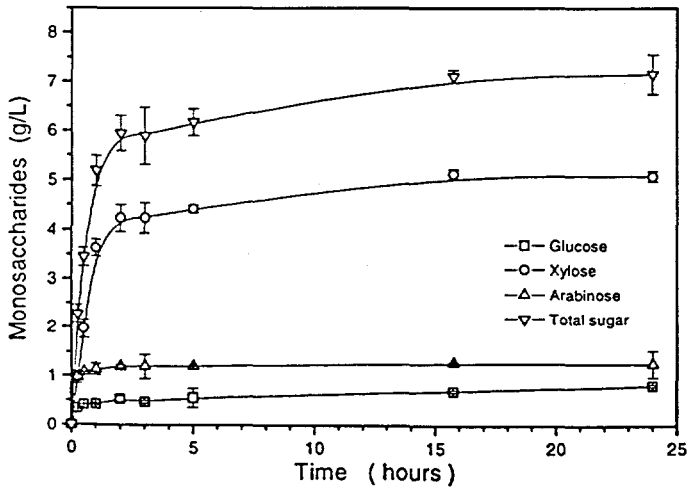

Figure 4. Monosaccharide yield after enzymatic hydrolysis of wet-oxidized wheat straw hemicellulose using the VTT-enzyme mixture as a function of reaction time (Conditions: 50°C, pH 5.0, 10 µL enzyme, 240 µL substrate (B91)).

4.3 Effect of pH-Value

Like other proteins, enzymes are amphoteric molecules. The charge of the amino acids varies with the pH-value of the environment according to their dissociation constants. A change in pH-value might affect the charges and configuration of the active site and thereby change the activity, structural stability, and/or solubility of the enzyme. Hence, the purpose of a pH-optimization is to identify at which pH-value the enzyme-active site has the best configuration resulting in optimal hydrolysis. Generally, the different xylanolytic enzymes differ in their optimal pH-values, but the majority lies within 4-7 (Viikari *et al.*, 1994). The enzymatic hydrolysis of wet-oxidized wheat straw was carried out at different pH-values (**Figure 5**). No distinct optimum was observed, however, a pH-value between 4.8 and 5.0 appeared to be slightly better, in accordance with Tenkanen *et al.* (1992a).

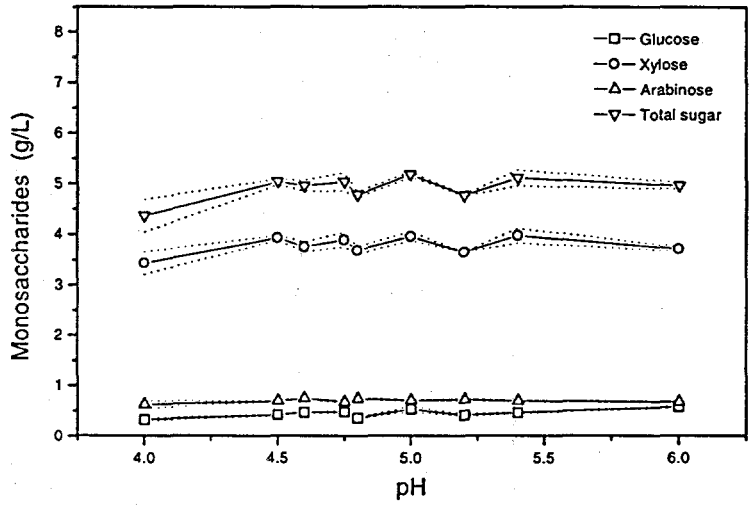

Figure 5 Monosaccharide yield after enzymatic hydrolysis of wet-oxidized wheat straw hemicellulose using the VTT-enzyme mixture as a function of the pH-value. (Conditions: 45°C, 24 hours, 10 µL enzyme, 240 µL substrate (B58)). The dotted lines indicate the deviation encounted during hydrolysis and analysis.

In order to investigate the extent to which the optimal pH-value was influenced by the composition of the substrate, enzymatic hydrolysis using a different wet-oxidized wheat straw (B91) (**Appendix A**) as substrate was performed (**Figure 6**). The hydrolysis of this substrate gave significantly higher sugar yields (about 6 g/L) than that of B58 (**Figure 5**). Xylose accounted for about 75% of the total sugar analyzed. The pH optimum was independent of the composition of the wet-oxidized substrate, whereas the total monosaccharide yield varied due to the different conditions giving different amount of solubilized hemicellulose and possibly also a different degree of precipitation during storage. The sugar yield from the hydrolysis of B58 was very low (only 5 g/L) compared to what was achieved by using another (B89) (more than 7 g/L)

(**Figure 7**), indicating a mishap in the pretreatment of this substrate (B58).

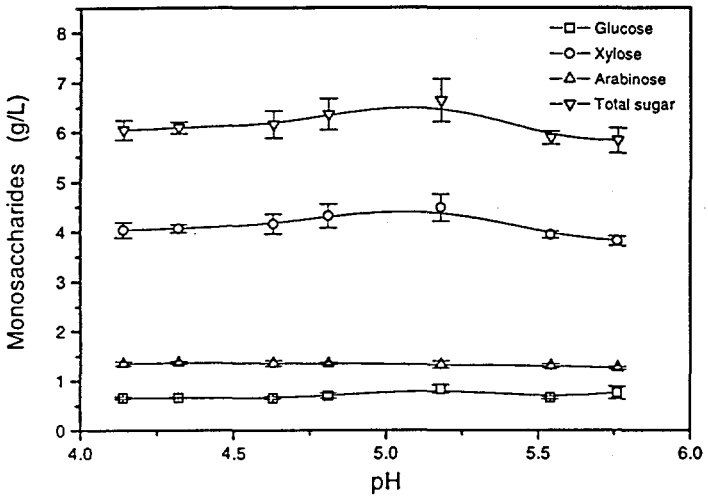

Figure 6. Monosaccharide yield after enzymatic hydrolysis of wet-oxidized wheat straw hemicellulose using the VTT-enzyme mixture as a function of the pH-value (Conditions: 45°C, 24 hours, 10 µL enzyme, 240 µL substrate (B91)).

To evaluate the effect of the prolonged incubation time of 24 hours on the lack of a distinctive optimum, an incubation time of 2 hours was applied (**Figure 7**). No clear optimum was identified even at this shorter incubation time; however, the xylose yield dropped gradually at a pH-value above 5.2, which is in accordance with low activity of xylanase at high pH-values (**Table 2**) (Angelo *et al.*, 1997).

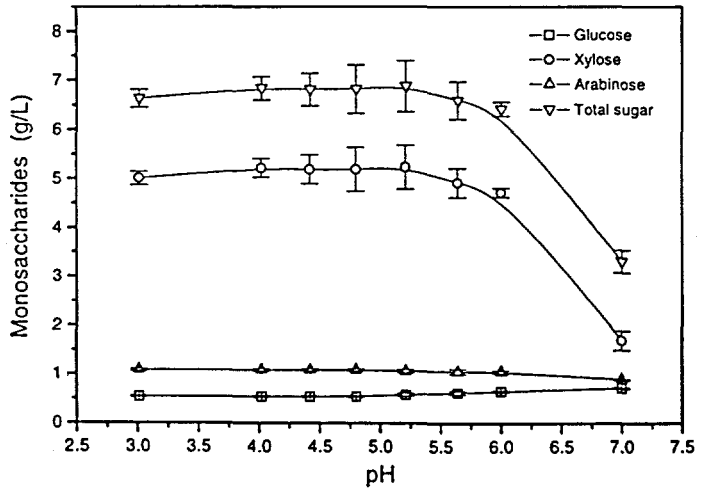

Figure 7. Monosaccharide yield after enzymatic hydrolysis of wet-oxidized wheat straw hemicellulose using the VTT-enzyme mixture as a function of the pH-value (Conditions: 50°C, 2 hours, 10 µL enzyme, 240 µL substrate (B89)).

Although a distinctive pH-optimum could not be identified (**Figure 7**), a hydrolysis at a pH-value between 4.8 and 5.2 seemed to give a better hydrolysis. Hence, a pH-value of 5 was chosen as the most suitable level for further investigations. The lack of a distinctive optimum (**Figures 5-7**) might be due to diversity in optimal pH-value for the different xylanolytic enzymes (Kormelink, 1992). Such a distinctive optimum might not exist for a mixture of different enzymes. For example, one specific xylanase might show high activity at low pH and low activity at high pH, whereas the opposite effect might be true for other xylanases.

4.4 Effect of Temperature

The rate of enzymatic reactions is strongly influenced by temperature. As temperature is raised the reaction rate increases until the enzymes reach their maximum temperature; following this the enzymes will undergo confirmation alteration resulting in a decrease or complete loss of activity caused by the increasing temperature.

For practical reasons the temperature optimization was carried out under two different conditions. By employing a different setup of the enzymatic hydrolysis, experiments at higher temperatures could be carried out. Additionally, the influence of the reaction time on the rate of hydrolysis was studied by using a considerably shorter hydrolysis time. The experiments were carried out at hydrolysis times of 24 hours (**Figure 8**) and 2 hours (**Figure 9**).

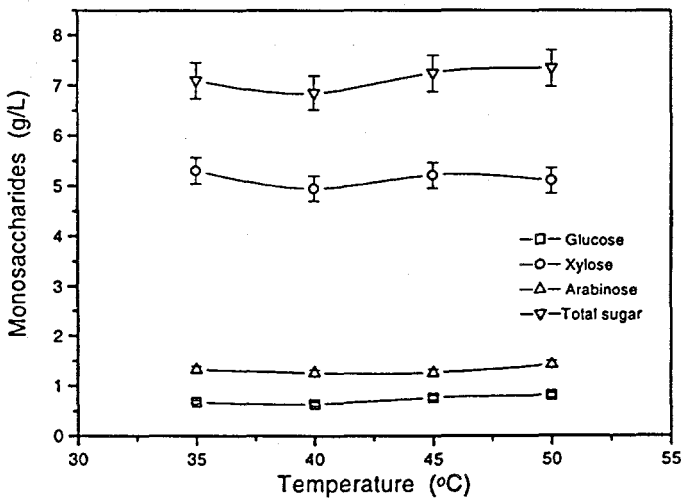

Figure 8. Monosaccharide yield after enzymatic hydrolysis of wet-oxidized wheat straw hemicellulose using the VTT-enzyme mixture as a function of the reaction temperature (Conditions: 24 hours, pH 5.0, 10 µL enzyme, 240 µL substrate (B91)).

By using the longer time, no distinct temperature optimum could be observed as all applied temperatures gave about the same release of monosaccharides. However, by using the shorter reaction time of 2 hours, a very distinct temperature optimum was observed at 50°C (**Figure 9**).

This indicates that after a 24-hour hydrolysis, the hydrolysis reaction will have reached equilibrium even when the reaction rate and temperature were not optimal.

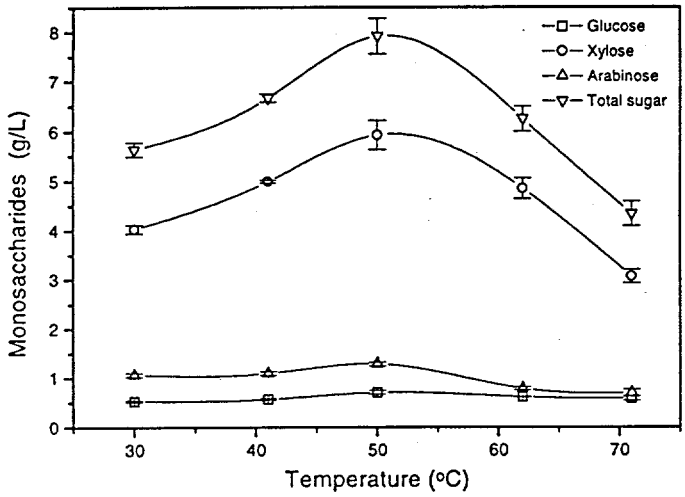

Figure 9. Monosaccharide yield after enzymatic hydrolysis of wet-oxidized wheat straw hemicellulose using the VTT-enzyme mixture as a function of the reaction temperature (Conditions: 2 hours, pH 5.0, 10 µL enzyme, 240 µL substrate (B89)).

4.5 Overall Discussion

The enzymatic hydrolysis was compared with the previously optimized acid hydrolysis on the same wet-oxidized wheat straw hemicellulose using the standard procedure developed at Risø National Laboratory (Bjerre *et al.*, 1996a). The overall monosaccharide yield from enzymatic hydrolysis was only 82% of that of the acid hydrolysis, but the ratio of the two hydrolyses was different for the different monosaccharides (**Table 5**). The yield of glucose was similar for the enzymatic and acid hydrolyses probably due to excess cellulase activity (**Table 3**) in the VTT-enzyme mixture relatively compared to the amount of solubilized cellulose (glucose) in the substrate. On the other hand, the yields of xylose and arabinose seemed to be closely linked yielding the same ratio between the two types of hydrolyses. This agrees with xylose and arabinose belonging to the same polymeric arabinoxylan, whereas glucose most probably derives from solubilized cellulose (Schmidt *et al.*, 1996).

The low yield of the monosaccharides deriving from the hemicellulose (only 80%) (**Table 5**) might be due to missing/limiting hemicellulase activities in the VTT-enzyme mixture needed for complete degradation of the solubilized wheat straw hemicellulose from wet-oxidation pretreatment. The specificity of the enzymes is primarily linked to the producing microorganism and secondly to the growth medium through induction (Faulds & Williamson, 1993). As the VTT-enzyme mixture was produced on a different media than the hydrolysis substrate all the necessary enzymes for complete hydrolysis might not have been induced

and produced, which could lead to missing or very low activities of some essential enzymes. If an enzyme mixture were produced on solubilized wheat straw hemicellulose from wet-oxidation pretreatment the enzymes necessary for complete hydrolysis might be formed with satisfying activity. Such an enzyme mixture should act much more specifically on the hemicellulose present in the pretreated wheat straw and give a better hydrolysis than the applied VTT-enzyme mixture. An investigation into the composition of oligosaccharides after the enzymatic hydrolysis might reveal which specific enzyme activities are missing or limiting, or whether or not inhibitors are present in the wet-oxidized substrate.

Table 5. Comparison of the monosaccharide yield from wet-oxidized wheat straw hemicellulose (B91) by using acid hydrolysis (4% w/v sulfuric acid, 120°C, 10 minutes) and enzymatic hydrolysis (VTT-enzyme mixture, volume-ratio = 0.042, pH = 5.0, 45°C, 24 hours).

Monosaccharides	Acid hydrolysis (g/L)	Enzymatic hydrolysis (g/L)	Sugar Ratio[a] (%)
Glucose	0.74	0.77	103
Xylose	6.47	5.22	81
Arabinose	1.59	1.26	80
Total sugar	8.81	7.25	82

a: The monosaccharide yield ratio of enzymatic to acid hydrolysis.

Due to the complexity of the substrate (**Figure 1**) and the well-documented synergistic effects of the hemicellulosic enzyme (Biely *et al.*, 1986; Faulds & Williamson, 1991; Tenkanen *et al.*, 1995a; Thomson, 1993) a limited activity of one enzyme will probably have a considerable impact on the overall yield of monosaccharides. The β-xylosidase is a key enzyme in the overall hydrolysis of hemicellulose (xylans) to xylose being responsible for the hydrolysis of xylotriose and xylobiose (Viikari *et al.*, 1994). The activity of this enzyme is relatively low in the VTT-enzyme mixture (**Table 3**), and a complete hydrolysis of the solubilized hemicellulose from wet oxidation might require a higher β-xylosidase activity. However, according to the experiments the overall yield of monosaccharides is not enhanced significantly with a larger enzyme dosage (**Figure 3**), which indicates a sufficient level of activity in the VTT-enzyme mixture.

Although, *T. reesei* is known for its capability to produce a vast variety of different xylanolytic enzymes, it has not been found to produce an esterase able to liberate ferulic acid from wheat straw xylan (Tenkanen *et al.*, 1991). On the other hand, three different types of feruloyl esterases have been isolated from *A. niger* (Faulds & Williamson, 1993). If wet-oxidized hemicellulose contain feruloyl groups on the sugar molecules the hydrolysis with the VTT-enzyme mixture might be hindered. However, this needs further investigation.

If specific side-chain-cleaving enzymes are missing, the sites for β-xylosidase and endo-1,4-β-xylanase might be blocked by side-groups leading to substituted xylo-oligomers. However, during wet oxidation most of the xylan-linked acetyl, feruloyl and p-coumaroyl side-groups

will most likely be hydrolyzed due to the alkaline and oxidizing conditions of the process, which is known to attack ester bonds (McGinnis *et al.*, 1983; Schmidt *et al.*, 1998). The alkaline conditions are known to promote the deacetylation of xylan enhancing the yield of the subsequently enzymatic hydrolysis (Kong *et al.*, 1992). Therefore, the subsequent enzymatic degradation of the xylan-backbone should not be inhibited by side-groups. This is presently being investigated.

By removing side-groups from the xylan backbone during the wet-oxidation process, the hemicellulosic polymers can interact with each other by forming hydrogen bonds (Andrewartha *et al.*, 1979). The solubilised hemicellulose has been observed to partly precipitate during storage at cold conditions (data not shown). This precipitate might be cross-bonded xylan explaining the diminish enzymatic digestibility of the present hemicellulose. The structure of the solubilized hemicellulose is presently under investigation in order to identify the bottleneck in the enzymatic hydrolysis.

The accessibility of the hemicellulose to hydrolytic enzymes is inversely correlated to the particle size (Converse, 1993). Although the wet-oxidation process reduces the particle size significantly this might not be sufficient to make the hemicellulose completely accessible, and a small amount of insoluble particles might reduce the overall yield.

Even though the enzymatic hydrolysis gave a lower quantification than acid hydrolysis it offers several advantages over acid hydrolysis. Depending on the application of the hydrolyzate, the further processing, both at analytical and industrial scales, will be less laborious by using enzymatic hydrolysis than by using acid hydrolysis without the risk of sugar degradation.

5 Conclusions

A method was developed for quantifying monosaccharides from solubilized wheat straw hemicellulose by enzymatic hydrolysis using a concentrated enzyme mixture prepared at VTT Biotechnology and Food Research by mixing four commercial enzyme preparations. Various hydrolysis parameters were studied such as enzyme loading, time, pH-value, and temperature in order to optimize the enzymatic hydrolysis. The minimal enzyme-substrate-volume-ratio needed to ensure maximal hydrolysis was about 0.042. Even though a hydrolysis time of 16 hours was sufficient, a reaction time of 24 hours was chosen in order to ensure equilibrium and total quantification. Already after about 2-5 hours of hydrolysis nearly all the sugars had been released from the polymer (about 90%).

By using 24 hours hydrolysis, no significant variations in the amount of released monosaccharides were observed for the temperature and pH-value. However, by using a shorter hydrolysis time of 2 hours, a very distinct temperature optimum was observed at 50°C. Even with this short hydrolysis time, no distinct optimal pH-value was identified. However, the monosaccharide yield decreased at pH-values above 5.2. An enzyme-substrate-volume-ratio of 0.042, a pH-value of 5.0, and a temperature of 50°C were chosen as the best hydrolysis conditions due to an improved monosaccharide yield. The hydrolysis time was set at 24 hours to ensure total quantification.

The enzymatic hydrolysis was compared to the acid hydrolysis standard method for quantification of solubilized hemicellulose developed at Risø National Laboratory (Bjerre et al., 1996a). Despite efforts to obtain a efficient enzymatic hydrolysis, it was impossible to obtain the same sugar yields by using enzymatic hydrolysis as by acid hydrolysis. The overall yield from the former was only 85% of that of the latter. The glucose yield was approximately the same for the two hydrolysis methods, probably due to high cellulase activity in the VTT-enzyme mixture and low amount of cellulose or other glucose containing material in the substrate. For xylose and arabinose the enzymatic hydrolysis gave only 80% of that of the acid hydrolysis. As the pentoses existed mainly as complex polymers their degradation requires many different enzymes, some of which might be missing from the VTT-enzyme mixture. Furthermore, the removal of side-chains from the xylan backbone during the wet-oxidation pretreatment process might enable the hemicellulosic polymers to interact and precipitate, hence, reducing the enzymatic digestibility of the hemicellulose.

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Appendix A: Materials

A1 Substrate

- The wheat straw was grown at Risø National Laboratory in 1993.
- After harvesting, the straw was dried and ground to less than 5 mm size.
- The composition of the untreated wheat straw is stated in **Table A1.**
- The substrate used for optimizing the enzymatic hydrolysis was produced by wet oxidation in a specially designed loop-autoclave constructed at Risø National Laboratory (Bjerre *et al.*, 1996b).
- The wet-oxidation conditions are given in **Table A2.**
- After the pretreatment, the suspension was filtered to separate the solid fraction from the liquid fraction.
- The filtrate containing the dissolved hemicellulose was employed in the hydrolysis study.

Table A1. The fiber composition (%w/w) of the untreated wheat straw.

NCWM[a]	Hemicellulose[b]	Cellulose[b]	Total Lignin[b]	Ash[b]	Convertible Cellulose[c]
18.8	32.8	38.0	8.9	1.4	12.9

a: NCWM = non cell wall materials (proteins, pectin etc.)
b: based on dry mass of wheat straw
c: based on cellulose in wheat straw

Table A2. Wet-oxidation conditions for hemicellulose solubilization.

Sample code	Straw (g/L)	Na_2CO_3 (g/L)	O_2-pressure (bar)	Temperature (°C)	Reaction time (minutes)
B58[a,b]	60	6.5	12	185	15
B89[b]	60	6.5	12	185	15
B91	60	6.5	6	185	15

a: Significantly lower monosaccharide yield in this pretreatment experiment.
b: Optimal wet-oxidation conditions for wheat straw from 1993 (Schmidt & Thomsen, 1998)

A2 Enzymes

- The concentrated enzyme mixture, with the enzyme activities given in **Table A3**, was prepared at VTT Biotechnology and Food Research, Finland, by combining commercial cellulase (Econase, Alko Ltd., Finland), xylanase (Ecopulp, Alko Ltd., Finland), mannanase (Gamanase, Novo Nordisk A/S, Denmark) and β-glucosidase (Novozym, Novo Nordisk A/S, Denmark) preparations.
- The mixture of the four commercial enzyme preparations was gel filtrated though a P-6 column (Biorad, USA) in order to remove contaminating sugars (Tenkanen *et al.*, 1995b).

Table A3. The activities in the VTT-enzyme mixture (Data provided by VTT Biotechnology and Food Research, Finland).

	Enzyme	Activity	Units
FPU[a]	FPase (Cellulase)	49	U/mL
HEC[b]	Endo-glucanase	4850	nkat/mL
XYL	Xylanase	5360	nkat/mL
MAN	Mannanase	10250	nkat/mL
β-Glu	β-Glucosidase	2050	nkat/mL
β-Xyl	β-Xylosidase	240	nkat/mL
β-Man	β-Mannosidase	71	nkat/mL
α-Ara	α-Arabinofuranosidase	1000	nkat/mL
α-Glur	α-Glucuronidase	2	nkat/mL
α-Gal	α-Galactosidase	240	nkat/mL

a: Filter Paper Activity Units
b: HEC: Hydroxyethylcellulose

Appendix B: Enzymatic Hydrolysis

B1 Materials

B1.1 Buffer

0.1 M acetic acid:

- 2.85 mL acetic acid was added to a 500 mL volumetric flask.
- The total volume was made up to 500 mL with deionized water.

0.1 M sodium acetate buffer:

- 4.10 g sodium acetate was dissolved in 500 mL deionized water.
- pH was adjusted by titration with the 0.1 M acetic acid.

B2 Procedure

B2.1 General Enzymatic hydrolysis

A 1-mL reaction mixture was produced as follows:

- 750 µL of 0.1 M acetate buffer (pH 5.0) was added to an Eppendorf tube.
- 240 µL of wet-oxidized wheat straw substrate was added to the tube.
- Then 10 µL of the VTT-enzyme mixture was added to the tube.
- The timer was started.
- The Eppendorf tubes were mounted in the specially designed rack.
- The rack with the tubes was then placed in a heated lab shaker (Adolf Kühner AG).
- The conditions for the shaker were set to 45°C and 120 rpm.
- The reaction mixture was incubated for 24 hours.
- The reaction was stopped by heating at 100°C for 5 minutes.
- The reaction mixtures were centrifuged at 14,000 rpm for 5 minutes (Sorvall RMC 14).
- 750 µL of supernatant was transferred to a 10 mL plastic test tube.
- 1 mL of deionized water was added
- The monosaccharide composition was analyzed on HPLC.

B2.2 Enzyme-substrate-volume-ratio

A 1-mL reaction mixture was produced as follows:

- 200-245 µL of wet-oxidized wheat straw substrate was added to the 750 µL buffer (pH 5.0) in the Eppendorf tube.
- Then 5-50 µL of the VTT-enzyme mixture was added to make up a total volume of 1.00 mL.
- The timer was started.
- The hydrolysis was carried out as described in **B2.1.**

B2.3 Reaction time

A 1-mL reaction mixture was produced as follows:

- The reaction mixture was made as described in **B2.1.**
- The timer was started.
- The tubes were mounted as described in **B2.1.**

- The conditions for the shaker were set to 50°C and 120 rpm.
- The reaction mixture was incubated for variable time period (0.25-24 hours).
- Further handling was as described in **B2.1.**

B2.4 pH-value

A 1-mL reaction mixture was produced as follows:

- 750 µL of 0.1 M acetate buffer with various pH-values (**B1.1**) was added to an Eppendorf tube.
- 240 µL of wet-oxidized wheat straw substrate was added to the tube.
- Then 10 µL of the VTT-enzyme mixture was added to the tube.
- The timer was started.
- The tubes were mounted as described in **B2.1.**
- The conditions for the shaker were set to 45 or 50°C and 120 rpm.
- The reaction mixture was incubated for 2 or 24 hours.
- Further handling was as described in **B2.1.**

B2.5 Temperature

A 1-mL reaction mixture was produced as follows:

- The reaction mixture was made as described in **B2.1.**
- The timer was started.
- The Eppendorf tubes were mounted in the specially designed rack.
- For temperatures at 50°C and below:
    - The rack with the tubes was placed in a heated lab shaker.
    - The conditions for the shaker were set from 30 to 50°C and 120 rpm.
    - The reaction mixture was incubated for 24 hours.
- For temperatures above 50°C:
    - The temperature of a water bath was set at different levels (30-70°C).
    - The rack with the tubes was placed in the pre-heated water bath.
    - The rack was shaken at 120 rpm.
    - The reaction mixture was incubated for 2 hours.
- Further handling was as described in **B2.1.**

Appendix C: HPLC Analysis

C1 Equipment

Pump:	Knauer HPLC-pump, model 364.00	
Columns:	Biorad Guard Column no. 125-0129	
	Biorad Aminex HPX-87H Column	
Run parameters:	Maximum pressure 10 mPa	
	Maximum flow rate 0.2 mL/min. at room temperature	
	Maximum flow rate 0.6 mL/min. at 63°C	
	Maximum temperature 65°C	
	pH 1-3	
Detector:	Knauer Differential Refractometer	
Integrator:	HP 3395	
Integrator set-up:	Zero = 10	Reset of base line
	ATT2 = 0	Attenuation. Set of range of peak heights
	CHT SP = 1.0	Chart speed
	AR REJ = 0	Threshold for rejection of area
	THRSH = 3	Threshold for rejection of peaks
	PK WD = 0.50	Expected peak width at half height
Integrator time:	5.000 INTG = 2	Set base line at all valleys
	7.000 INTG = 8	Turn on start/stop marks
	15.000 STOP	Stop integration
Solvent:	0.004 M H_2SO_4	

C2 Start-Up Procedure

- The flow [P4] (**Figure C1**) was turned off by lowering the flow rate gradually by 0.1 mL/min. allowing the pressure to stabilize.
- The solvent tube was removed from the solvent and wrapped in aluminum foil.
- The solvent was treated in an ultrasonic bath for 5 minutes.
- Meanwhile the *pump* [P16] was washed with 5 mL water.
- The *detector* reference cell [D9] was filled with treated solvent with a 10 mL syringe by removing one of the ends of the plastic tube and injecting (5-10 mL) solvent until all the air was removed.
- The solvent tube was placed in the treated solvent and the *detector* outlet tube [D10] was connected to the waste container.
- The valve [P18] on the *pump* was turned left and a beaker was held under the outlet [P21].
- The purge button [P13] was pressed for approximately 2 minutes to remove trapped air from the system.
- The valve [P18] was closed by turning to the right.
- In the case the *pump* did not start ([P6] will then show a red light) the pump was reset by switching the reset button [P2].
- The flow rate was gradually raised to 0.2 mL/min. and the column oven was turned on.
- The column was heated to 63°C before the flow rate gradually was raised to 0.6 mL/min.
- To allow the HPLC system to stabilize the HPLC was left running with eluent for 15 minutes.

- The *detector* was reset to 000 mV by pressing the FINE button [D5] and turning the COARSE knob [D4].

C3 Sample Run

- The integrator set-up parameters was checked by pressing [LIST] [LIST] on the *integrator.*
- The integrator time parameters was checked by pressing [LIST] [TIME] [ENTER] on the *integrator.*
- A 1 mL syringe was washed twice with water and once with the sample.
- A 0.7 mL sample was taken.
- The trapped air in the top of the syringe (piston end) was removed by tapping the on the syringe.
- The *injector valve* was turned to the [LOAD] position and the sample was loaded into the 20 µL loop by injection through the membrane (**avoiding injection of air**).
- The syringe was left in the membrane.
- The *injector valve* was then turned to the [INJECT] position without touching the syringe. The sample in the loop was injected on to the column.
- Simultaneously the [START] button was pressed on the *Integrator.*
- The analysis time was 15 minutes.

C4 Shut-Down Procedure

- The flow rate was gradually lowered [P5] to 0.2 mL/min.
- The column oven was switched off.
- The *detector* outlet tube [D10] was placed in the solvent flask for recycling of eluent.
- The eluent was running continuously through the system in order to avoid the column to dry out.
- The *pump* [P16] was washed with 5 mL of water.

C5 Reagents

C5.1 Eluent

- 0.004 M sulfuric acid: 213 µL 95-97% H_2SO_4 was added to a 1 L volumetric flask containing some water and the volume was made up to 1 L with water.
- The eluent was filtered through a 0.45 µm filter.

C5.2 Sugar Standards

- 6 mM glucose and arabinose standard: 0.0540 g glucose and 0.0450 g arabinose was transferred to a 50 mL volumetric flask. Water was added to a total volume of 50 mL.
- 6 mM xylose standard: 0.0450 g xylose was transferred to a 50 mL volumetric flask. Water was added to a total volume of 50 mL.

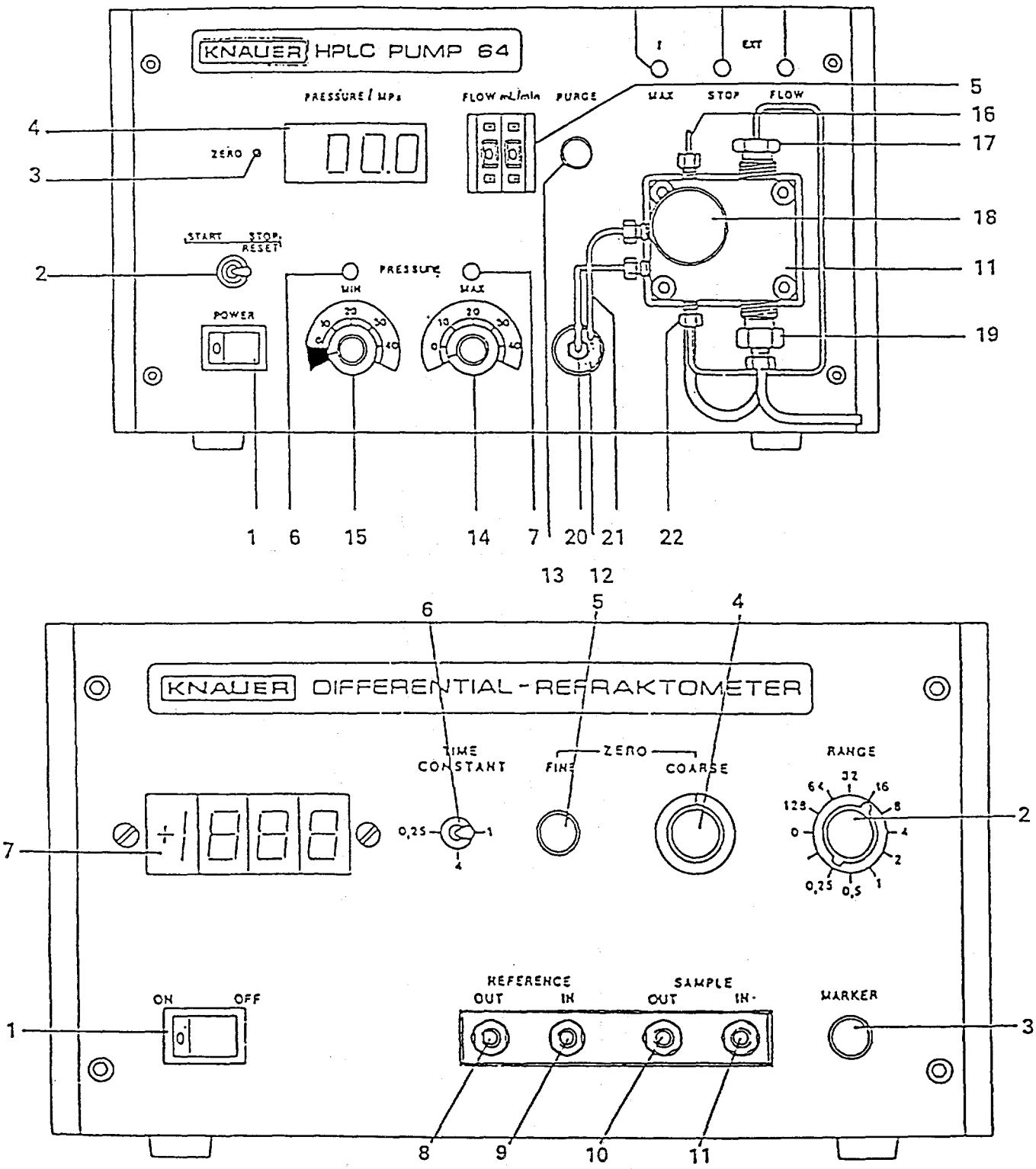

Figure C1. The control panel of the pump [P] (Knauer, model 364.00) and the detector [D] (Knauer Differential Refractometer) used in this study.

Appendix D: Calculations

D1 Notation

Ratio = Volume ratio of enzyme/substrate in the hydrolysis mixture.
DF = Dilution factor
= The mixture is diluted after hydrolysis prior to HPLC analysis.
CF = Conversion factor
= Calculated on basis of HPLC standard curve for particular sugar.
Area = The area of the particular sugar peak detected on the HPLC.

C_S(HPLC) = Sugar concentration as measured on HPLC (g/L).
C_S(hydrolysis) = Sugar concentration as measured in hydrolyzate (g/L).
C_S(substrate) = Sugar concentration as measured in the substrate (g/L).
C_S(enzyme) = Sugar conc. as measured in VTT-enzyme mixture (g/L).

M_S = Molecular weight of sugar (g/mole)

m_S(hydrolysis) = Mass of sugar in the hydrolyzate (g).
m_S(substrate) = Mass of sugar in the substrate (g).
m_S(enzyme) = Mass of sugar in the VTT-enzyme mixture (g).

V(hydrolysis) = Total volume of hydrolysis mixture (L).
V(substrate) = Volume of substrate (L).
V(enzyme) = Volume of VTT-enzyme mixture (L).

D2 Calculations

$C_S(\text{HPLC}) = \text{Area} \cdot CF \cdot M_S$
$C_S(\text{hydrolysis}) = C_S(\text{HPLC}) \cdot DF$

Mass balance:
$m_S(\text{substrate}) = m_S(\text{hydrolysis}) - m_S(\text{enzyme})$
$C_S(\text{substrate}) \cdot V(\text{substrate}) =$
$C_S(\text{hydrolysis}) \cdot V(\text{hydrolysis}) - C_S(\text{enzyme}) \cdot V(\text{enzyme})$

$$C_S(\text{substrate}) = \frac{C_S(\text{hydrolysis}) \cdot V(\text{hydrolysis}) - C_S(\text{enzyme}) \cdot V(\text{enzyme})}{V(\text{substrate})}$$

D3 Example of Calculation

To illustrate how C_S(substrate) was found an example of the calculations is shown below. The hydrolysis EH20 (see Table C1) was used to calculate the **xylose** concentration.

Ratio = 2/23 = 0.0870
M_S(xylose) = 150.1 g/mole
CF(xylose) = $0.349 \cdot 10^{-6}$ mM/area

C_S(enzyme):

DF = 10 · 4
Area = (2,112,464 + 2,144,616)/2 = 2,128,540

$C_S(HPLC) = Area \cdot CF \cdot M_S$
$= 2{,}128{,}540 \cdot 0.349 \cdot 10^{-6}$ mM · 150.1 g/mole · 10^{-3} mole/mmole
$= 0.112$ g/L

$C_S(enzyme) = C_S(HPLC) \cdot DF$
$= 0.112$ g/L · 40 = *4.460 g/L*

C_S(hydrolysis):

DF = 2.33
Area = (10,856,280 + 10,745,568)/2 = 10,800,924

$C_S(HPLC) = Area \cdot CF \cdot M_S$
$= 10{,}800{,}924 \cdot 0.349 \cdot 10^{-6}$ mM · 150.1 g/mole · 10^{-3} mole/mmole
$= 0.566$ g/L

$C_S(hydrolysis) = C_S(HPLC) \cdot DF$
$= 0.566$ g/L · 2.33 = *1.318 g/L*

C_S(substrate):

$$C_S(substrate) = \frac{C_S(hydrolysis) \cdot V(hydrolysis) - C_S(enzyme) \cdot V(enzyme)}{V(substrate)}$$

$$= \frac{1.318 \text{ g/L} \cdot 1000 \text{ µL} - 4.460 \text{ g/L} \cdot 20 \text{ µL}}{230 \text{ µL}} = 5.344 \text{ g/L}$$

Bibliographic Data Sheet **Risø-R-1042(EN)**

Title and author

Hydrolysis of Solubilized Hemicellulose Derived from Wet-Oxidized Wheat Straw by a Mixture of Commercial Fungal Enzyme Preparations

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ISBN	ISSN
87-550-2378-9	0106-2840

Department	Date
Plant Biology and Biogeochemistry	April 1998

Pages	Tables	Illustrations	References
40	8	10	65

Abstract (max. 2000 characters)

The enzymatic hydrolysis of the solubilized hemicellulose fraction from wet-oxidized wheat straw was investigated for quantification purposes. An optimal hydrolysis depends on factors such as composition of the applied enzyme mixture and the hydrolysis conditions (enzyme loading, hydrolysis time, pH-value, and temperature). A concentrated enzyme mixture was used in this study prepared at VTT Biotechnology and Food Research, Finland, by mixing four commercial enzyme preparations. No distinctive pH-value and temperature optima were identified after a prolonged incubation of 24 hours. By reducing the hydrolysis time to 2 hours a temperature optimum was found at 50°C, where a pH-value higher than 5.2 resulted in reduced activity. An enzyme-substrate-volume-ratio of 0.042, a pH-value of 5.0, and a temperature of 50°C were chosen as the best hydrolysis conditions due to an improved monosaccharide yield. The hydrolysis time was chosen to be 24 hours to ensure equilibrium and total quantification. Even under the best hydrolysis conditions, the overall sugar yield from the enzymatic hydrolysis was only 85% of that of the optimal acid hydrolysis. The glucose yield were approximately the same for the two types of hydrolyses, probably due to the high cellulase activity in the VTT-enzyme mixture. For xylose and arabinose the enzymatic hydrolysis yielded only 80% of that of the acid hydrolysis. As the pentoses existed mainly as complex polymers their degradation required many different enzymes, some of which might be missing from the VTT-enzyme mixture. Furthermore, the removal of side-chains from the xylan backbone during the wet-oxidation pretreatment process might enable the hemicellulosic polymers to interact and precipitate, hence, reducing the enzymatic digestibility of the hemicellulose.

Descriptors

WHEAT, STRAW, HEMICELLULOSE, PENTOSES, ENZYMATIC HYDROLYSIS, BIODEGRADATION, pH-VALUE, TEMPERATURE DEPENDENCE

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